6-1-1980

Use of gadolinium in 13C NMR studies

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USE OF GADOLINIUM REAGENTS IN ^{13}C NMR STUDIES

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JUNE, 1980

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF GRAPHS

LIST OF FIGURES

ACKNOWLEDGMENT

The author would like to thank Dr. T. C. Morrill for his time, patience, and guidance in this research, and Dr. Craig Van Antwerp for his guidance and valuable discussions.

Appreciation is extended to the Rochester Institute of Technology for support of this work.

ABSTRACT

The addition of the paramagnetic reagents gadolinium(18-crown-6)nitrate, gadolinium(dicyclohexano-18-crown-6)nitrate, and gadolinium nitrate pentahydrate to each of the substrates ethylbenzene, and thymidine has been studied in an attempt to obtain quantitative ^{13}C spectra. These reagents reduce the nuclear Overhauser effect and shorten the spin-lattice relaxation times of the carbons of these substrates.

HISTORICAL

It has been reported[1,2] that proton-decoupled Fourier transform ^{13}C spectra show signals which vary greatly in their intensities; i.e., a quantitative relationship between intensity and the number of ^{13}C nuclei does not exist. Instrumental considerations and characteristics of the molecule contribute to these intensity variations. Instrumental effects such as insufficient rf power to irradiate all the nuclei equally effectively and insufficient computer memory (data points) to completely define all the peaks[2] may be overcome, so only molecular characteristics will be further described. These include variations in the spin-lattice relaxation times and differences in the nuclear Overhauser enhancement for various carbons in the molecule.

Irradiation of the protons in a sample disturbs the Boltzmann distribution of the ^{1}H energy level populations (at equilibrium, the Boltzmann distribution describes a slight excess of nuclei in the lower spin state, which gives rise to the net absorption of energy in the radio frequency region). Saturation of the proton rf region equalizes the ^{1}H energy level population and creates a greater inequality of ^{13}C energy level populations by causing radiationless transitions by which nuclei in the upper spin state relax to a lower spin state.[2,3,12,17]

Since the Boltzmann distribution is disturbed when nuclei are irradiated, it is important to be aware of the time needed for a molecule's nuclei to return to the Boltzmann populations. Only when there is sufficient time after an rf pulse is applied, can all nuclei be treated equally. This relaxation occurs as an inverse exponential with respect to time

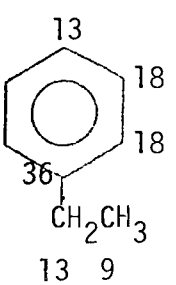
and is referred to as the spin-lattice relaxation time (T_1) when the process of return is by dissipation of the energy to the nucleus' surroundings. Various mechanisms contribute to spin-lattice relaxation, including dipole-dipole interactions, spin-rotation, quadrupolar, scalar, and chemical shift anistropy. The first two are usually the most important for organic molecules. Dipolar relaxation is usually an effective and efficient relaxation mechanism. For example, a carbon nucleus close to a nucleus with a magnetic spin, such as hydrogen, is efficiently relaxed because the interaction of the magnet moment of the carbon with the nearby hydrogen facilitates the relaxation. The hydrogen is especially efficient since it has a high magnetic moment relative to carbon. Spin-rotation relaxation arises from fluctuating magnetic fields due to movement of atoms within a molecule, for example, rotation of a methyl group. Quadrupolar relaxation is only important for nuclei with spins greater than one-half, such as ^{14}N and deuterium. These nuclei create an electric field gradient and provide an efficient relaxation mechanism for any neighboring nuclei and for themselves. Scalar and chemical shift anistropy generally do not affect carbon spin-lattice times to a significant extent. The possibility of contribution from chemical shift anistropy to ^{13}C relaxation need be considered only in highly-anisotropic molecules, such as $RC{\equiv}N$ or $R_2C{=}O$. Scalar coupling is important in molecules where a carbon atom is directly bonded to atoms such as bromine.[2]

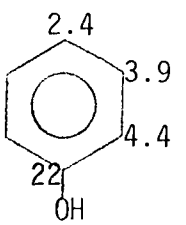
The Nuclear Overhauser effect (NOE) can be considered as an increase in ^{13}C signal intensities due to proton decoupling and the related disturbance in the Boltzmann population. Carbon nuclei react to equalization of ^{1}H energy level populations by changing their populations through relaxation mechanisms. This change results in an excess of nuclei in the

lower ^{13}C energy level relative to the Boltzmann distribution. More rf energy can be absorbed by the ^{13}C nuclei due to the larger population in the lower energy level and thus the signals will be more intense. The NOE, which has a theoretical maximum value of 2.988, and dipole-dipole relaxation are similar in that they are both facilitated by nearby hydrogens aiding the relaxation mechanism. In practice, the maximum NOE often is not realized due to the presence of a variety of relaxation mechanisms in a system.[3,4,5,6,8,10,14]

For quantitative peak intensities, wide ranges of spin-lattice relaxation times and NOE values must be considered. The effects of differing spin-lattice relaxation times can be overcome by introducing a pulse delay after each data acquisition period to ensure that virtually all nuclei completely relax. This usually occurs when the delay is more than five times the longest T_1. Experiments requiring many transients utilizing long pulse delays would take a considerable amount of time. It is not uncommon for these experiments to require two to five days of data acquisition. For example, if a system has relaxation times of five to twenty-five seconds, this would require 5 X 25 = 125 seconds as the minimum pulse delay. Therefore, a 4000-pulse experiment with a 125-second delay between each pulse would require almost six days to complete.

Following are some compounds with the spin-lattice relaxation times (in seconds) shown for each carbon[2],





9.3 68 13 23 9.8
$(CH_3)_3C\text{-}CH_2\text{-}CH(CH_3)_2$

NOE factors can be eliminated by utilizing a gated decoupling technique; that is, the proton decoupler is kept off during a pulse delay (5 X longest T_1, to ensure that the equilibrium populations have had time to re-establish) so when the pulse is applied the spin states have their normal equilibrium populations.[2] The proton decoupler is on only during the data acquisition.

In an effort to overcome long and variable T_1 and NOE factors and the related time-consuming experiments, LaMar proposed in the early 1970's the addition of a paramagnetic substance (substance with one or more unpaired electrons) to the NMR tube containing a solution of the substrate. Since the magnetogyric ratio of an electron is approximately 657 times greater than that of a proton, the presence of the unpaired electrons would lead to an efficient, and usually dominant dipole-dipole relaxation mechanism.[4]

Spin-lattice relaxation processes can be related as follows:

$$\frac{1}{T_1} = \frac{1}{T_1 d} + \frac{1}{T_1 e}$$

where: $\frac{1}{T_1}$ = observed relaxation time

$\frac{1}{T_1 d}$ = sum of diamagnetic relaxation terms

$\frac{1}{T_1 e}$ = Electron (paramagnetic) relaxation time due to electron nuclear relaxation mechanisms.

When a relaxation reagent is added, $T_1 e$ is very small, therefore, $1/T_1 e$ is large and $1/T_1 = 1/T_1 e$ since $1/T_1 e \gg 1/T_1 d$.

Paramagnetic substances described in the literature for quantitative ^{13}C-NMR studies include:

$Cr(acac)_3$[1,7,10,19], $Fe(acac)_3$[7], Cr^{+3} [4], $Cr(dpm)_3$[7], radicals[9], $Gd(fod)_3$[13], $Gd(dpm)_3$[5]; where:

(acac) = acetylacetonate

(dpm) = 2,2,6,6-tetramethyl-3,5-heptanedionato

(fod) = 1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedione

In summary, two feasible methods of quantitative ^{13}C analysis are presently commonly used, each with its own advantages and disadvantages:

(1) Long pulse delays and gated decoupling.

The advantage of this method is that it would require the addition of no foreign material (paramagnetic substance). The main disadvantage is the large amounts of time required.

(2) Addition of a paramagnetic substance.

The advantage here is that relatively short times are required and, therefore, it sometimes is more desireable as an analytical technique. Also, although the NOE is decreased (signal/noise is decreased), the decrease in T_1 permits the use of much faster pulsing rates with little or no pulse delays and therefore actually effecting a net increase in signal/noise for a given amount of experimental time. The dual disadvantages are: 1) it cannot be used with substrates that react with the paramagnetic substance, and 2) in various rigid molecules there may be sterically inaccessible carbon atoms shielded from contact with the relaxation agent.[1,5,6]

In the mid-1970's, experimentation indicated that upon addition of a paramagnetic substance, the T_1 values became equalized and shortened, whereas the NOE effect was reduced but wasn't fully eliminated. For organic molecules of molecular weight $\geq$ 200, NOE suppression is incomplete and variable at practical paramagnetic reagent concentration; this is due to efficient ^{13}C-^{1}H dipole-dipole relaxation which is able to successfully compete with electron-nuclear relaxation. In small molecules, rapid

molecular tumbling makes the ^{13}C-^{1}H dipole-dipole relaxation process inefficient.[5,14,15]

Applications relating to the effect of adding paramagnetic substances include:

(a) Quantitative analysis of syn-anti mixtures of oximes.[11]

(b) Shortening the usually long T_1 values of metal-bonded carbonyls and, therefore, decreasing experimental time. Long T_1 values are observed in these cases because no hydrogens are directly attached to the carbon to provide this dipole-dipole relaxation.[5,19] An example of long T_1 times of carbonyls is provided by the following keto-lactone:

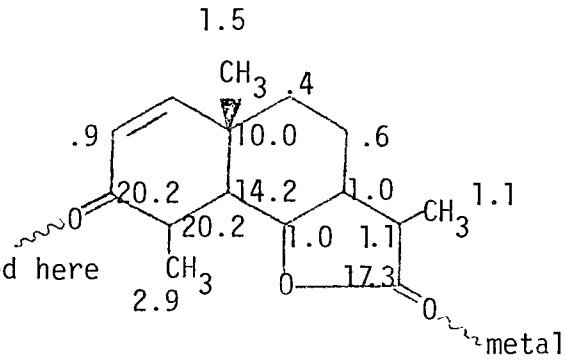
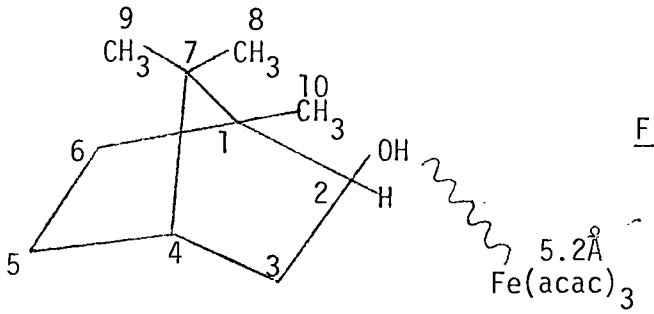


Fig. 1

(c) Use of paramagnetic ions in FT-^{13}C NMR biosynthetic studies to help overcome difficulties of detecting carbons that have low ^{13}C enrichment.[18]

(d) Quantitative studies of molecular conformations and conformational equilibria in organic solutions. Since the enhancement of the electron-nuclear dipole-dipole relaxation interaction is proportional to $1/r^6$ (r = distance between the observed nucleus and the metal ion) this technique is a useful source of structural conformation. No angular dependence occurs, in contrast with the situation for pseudocontact shifts in lanthanide reagent complexes. The individual spin-lattice relaxation terms for any

nucleus in a substrate complexed to a paramagnetic reagent depends on the time-averaged internuclear distance between that nucleus and the paramagnetic metal, nuclei close to the site of complexation have the shortest spin-lattice relaxation terms.[13,16] For example (Figure 2),



isoborneol

Fig. 2

(location of $Fe(acac)_3$, approximately 5.2Å from the -OH group and 3Å below the plane containing carbons 2,3,5,6).

$Fe(acac)_3$ will preferentially complex to the -OH group. Therefore, the carbons closest to the -OH group, C_1, C_2, and C_3, have particularly short T_1's ($\leq$ .3 seconds), whereas for remote carbons, C_4 through C_9, the T_1 exceeds .4 seconds. Table II[16]:

Spin-Lattice Relaxation Data
(For isoborneol, Fig. 2)

Carbon	$1/(T_1)$ or C_2/C_x	$(r_{Fe(acac)}^{-x}/r_{Fe(acac)_3}^{-C_2})^6$
1	2.9	3.7
2	--	--
3	3.6	2.5
4	7.5	9.1
5	11.4	11.4
6	7.5	7.5
7	11.4	9.8
8	9.0	8.3

Carbon	$1/(T_1)$ or C_2/C_x	$(r_{Fe(acac)}-x/r_{Fe(acac)_3}-C_2)^6$
9	45*	26*
10	2.2	2.4

*Since $1/(T_1)$ is a small difference between two large numbers, this value is subject to considerable error.

Quantitative Studies of Thymidine Dimerization

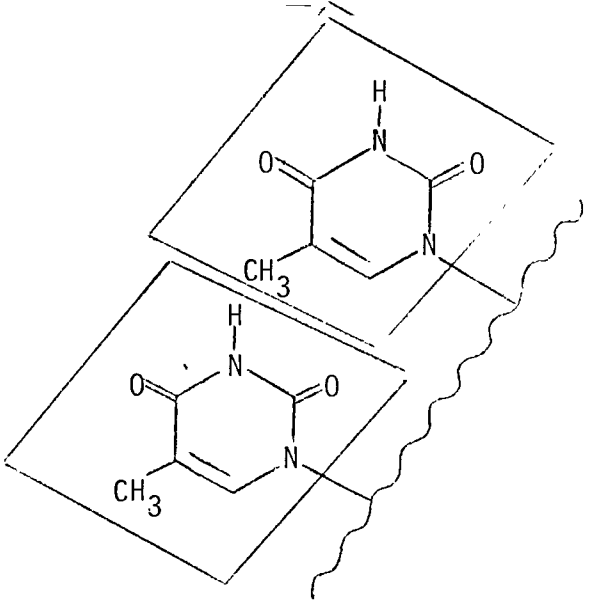
During the course of our studies of Gd-^{13}C NMR reagents, it became clear that a reasonable place to apply such reagents was to certain nucleoside systems. Here we discuss the background of the specific systems of interest to us.

Thymidine and thymine molecules dimerize when subjected to UV irradiation at 254nm. Much of the damage produced in various biological systems by UV light has been attributed to cyclobutane-type dimers formed between adjacent pyrimidines in DNA.[21,22,23]

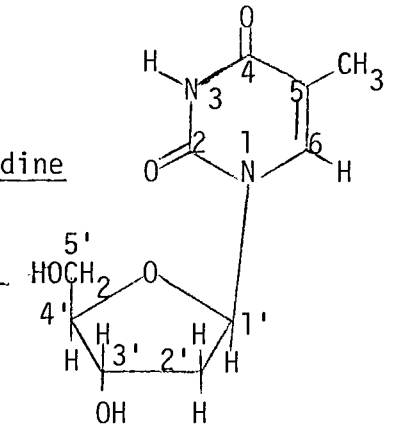
Intrastrand dimerization is expected to take place readily in nucleic acids since adjacent pyrimidines in a given DNA strand are properly aligned (see below and Figure 3). Intrastrand dimerization, that is bonding between two pyrimidines (one in each strand), could give rise to either cis-anti (Figure 5) or trans-anti (Figure 6) products.[25] Beukers and Berends (1961)[21,22] discovered that thymine in frozen solution irradiated with UV light (254nm) yielded a dimer in which two thymine molecules are joined by two covalent bonds, one between their number five carbon atoms and one between the number six atoms.* Re-irradiation of the dimer in liquid solution at the same wave-length has resulted in its conversion back to thymine suggesting that the monomer and dimer formation are wave-length dependent and photochemically reversible.[20]

---

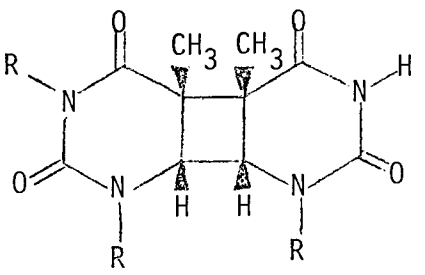
*By varying the conditions of thymidine and thymine molecules, only one dimer is produced (only the cis-syn (Figure 4) isomer is produced by UV irradiation of thymine in frozen aqueous solutions) or other adduct-type products are produced in addition to the cyclobutane dimers (Figures 3-6) as in frozen aqueous solutions of thymidine.[21,24]



Thymidine

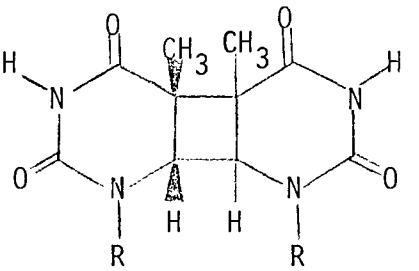


Four distinct isomeric cyclobutane type dimers can result from UV irradiation:[21,22,23]



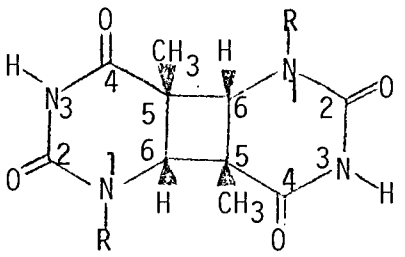
cis-syn (meso), predominant product

Fig. 3



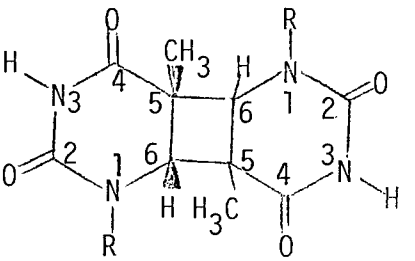
trans-syn (d,l), minor product

Fig. 4



cis-anti (d,l)

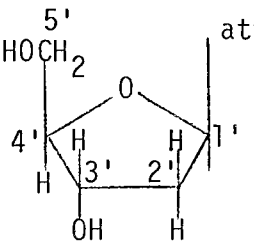
Fig. 5



trans-anti (meso)

Fig. 6

R = sugar group



attachment to base

Fig. 7

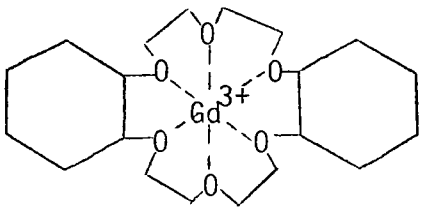
The purpose of this reasearch was to investigate the possibility of using the lanthanide metal gadolinium (a paramagnetic ion with seven unpaired electrons, $4f^7$) and its complexes for quantitative ^{13}C spectra in ethylbenzene and thymidine systems. Specifically, the gadolinium reagents were expected to induce quantitative signals for the ^{13}C spectra of thymidine and its dimer and thus allow accurate measurement of the monomer/dimer ratio by magnetic pulse resonance.

The in vivo formation of anti-parallel ring systems, that is cis-anti (Figure 5) and trans-anti (Figure 6) products is unlikely for steric reasons. It is expected that two adjacent rings of in vivo thymidine will come from the same general directions, that is, they will be attached to a common backbone with the R groups pointing in the same direction (pg. 10, left). Thus the syn isomers (with the R groups on a common side

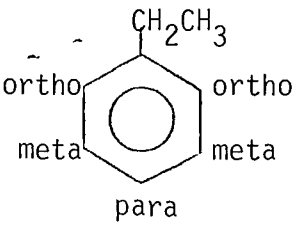
of the dimer system and the CH_3 groups on a common side of the cyclobutane system) are expected to be formed. The cis/trans notation refers to the relative geometry of the two methyl groups of a given isomer.

RESULTS AND DISCUSSION

Work carried out by a previous researcher (Dr. Alan Lindmark) was repeated. This consisted of quantitative incremental additions of gadolinium(dicyclohexano-18-crown-6)nitrate to ethylbenzene in an attempt to obtain quantitative integration of the ^{13}C signals for the substrate.



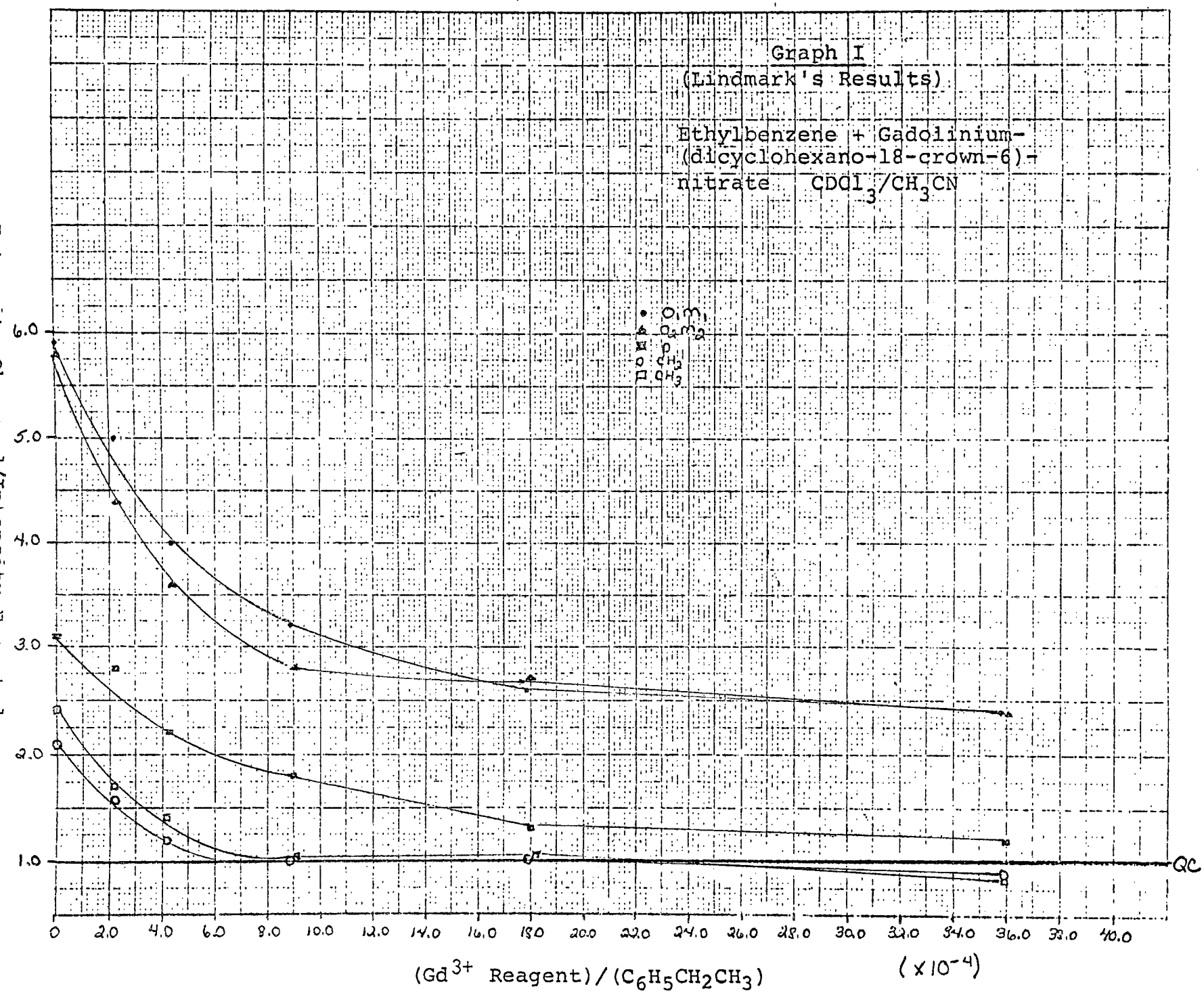
Gadolinium(dicyclohexano-18-crown-6)

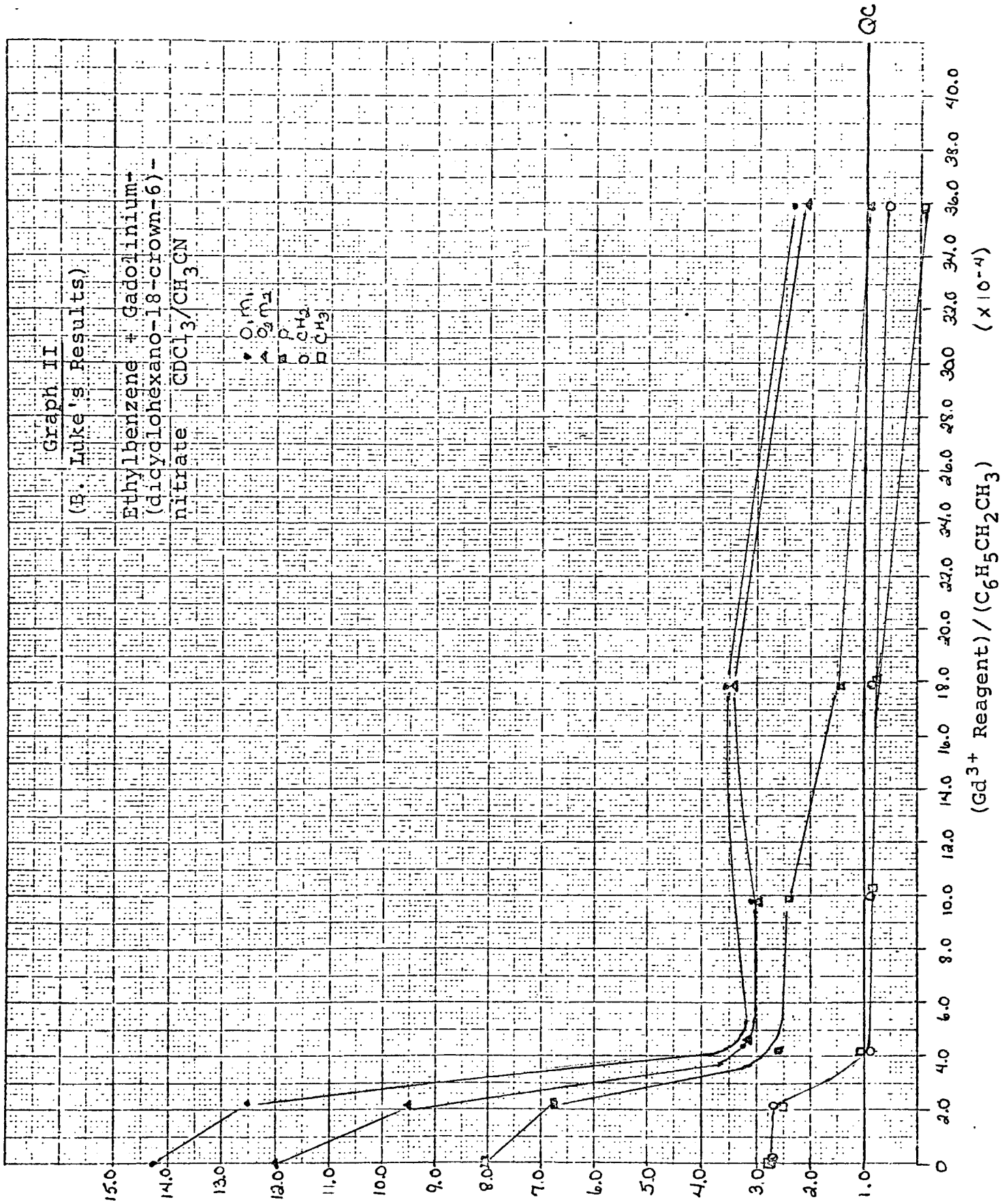


Ethylbenzene

Fig. 8

The binary solvent system used was $CH_3CH/CDCl_3$, as CH_3CN was needed to help dissolve the gadolinium complex. The carbon-13 spectrum was obtained, and a plot of the ratios of intensity observed to intensity expected for the ^{13}C signal of each carbon versus mole ratios of gadolinium reagent to ethylbenzene was obtained (See Graph I). (Intensity expected for one carbon is arbitrarily set as the intensity of the weakest signal in the spectrum; usually a quaternary carbon such as C-1 of ethylbenzene has the lowest intensity.) Thus, if all signals were quantitative, the intensity ratio, for example on Graph I, would equal the number of carbons for that signal. The ortho and the meta carbons of ethylbenzene could not be assigned with certainty since they differ by only 0.4ppm. Therefore, the notation o_1m_1 for one set of signals and o_2m_2 for the other set of signals was used. The literature does suggest the ortho carbons are


Graph I
(Lindmark's Results)
Ethylbenzene + Gadolinium-(dicyclohexano-18-crown-6)-nitrate CDCl3/CH3CN
Intensity Observed/Intensity Expected for One Carbon
(Gd3+ Reagent)/(C6H5CH2CH3) (x10-4)
QC
1.0
2.0
3.0
4.0
5.0
6.0
0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
22.0
24.0
26.0
28.0
30.0
32.0
34.0
36.0
38.0
40.0


Graph II
(B. Luke's Results)
Ethylbenzene + Gadolinium-(dicyclohexano-18-crown-6)-nitrate CDCl3/CH3CN
o, m1
o2 m2
p
o CH3
CH3
QC
(Gd3+ Reagent)/(C6H5CH2CH3) (x10-4)
Intensity Observed/Intensity Expected for One Carbon

further downfield than the meta carbons.[32]

Successful quantitative integration would be the case if the relative intensities of the o_1m_1 and o_2m_2 carbons reached two, and the quaternary (QC), the para, CH_2 and CH_3 carbons reach one as the result of addition of increased amounts of the relaxation reagent. The CH_3CN carbon integrations are also considered on Table I and are integrated with a different internal reference.

The columns of data marked (x), which are the result of our more recent experiments, indicate that upon increasing additions of gadolinium-(dicyclohexano-18-crown-6)nitrate, the ring carbons of ethylbenzene and CH_3CN become quantitative at $35.9 \times 10^{-4} = (Gd^{3+})/(C_6H_5CH_2CH_3)$ molar ratio. Our results (in the columns marked (X)) are qualitatively similar to Lindmark's earlier results (in the columns marked (+)). The apparent quantitative inconsistencies between columns (X) and (+) are possibly due to weighing errors as milligram amounts (1-16mg) of gadolinium complex were used.

Previous work[31] indicated that crown ether-lanthanide cation (III) metal complexes dissociate in polar solvents such as DMSO and CH_3CN, possibly allowing the CH_3CN to complex with the crown ether.

Gadolinium(18-crown-6)nitrate was also used as a relaxation reagent with substrate ethylbenzene in the same solvent system ($CH_3CN/CDCl_3$).

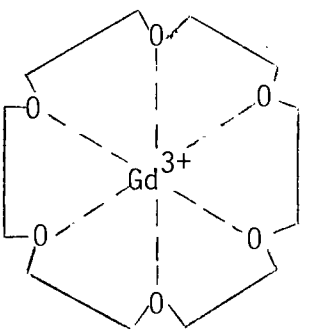


Fig. 9

Gadolinium(18-crown-6)

TABLE I

Relative Integrations for Carbons of CH_3CN and Ethylbenzene Treated with Gadolinium(dicyclohexano-18-crown-6)nitrate; $CDCl_3/CH_3CN$**

$\frac{(Gd^{3+})}{(C_6H_5CH_2CH_3)}$	0		2.24×10^{-4}		4.4×10^{-4}		8.98×10^{-4}		17.95×10^{-4}		35.9×10^{-4}	
	(+)*	(x)*	(+)	(x)	(+)	(x)	(+)	(x)	(+)	(x)	(+)	(x)
Carbon												
QC	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
o_1m_1	5.9	14.29	5.0	12.5	4.0	3.23	3.2	3.13	2.6	3.57	2.4	2.33
o_2m_2	5.8	12.0	4.4	9.5	3.6	3.2	2.8	3.0	2.7	3.4	2.4	2.12
p	3.1	8.0	2.8	6.75	2.2	2.58	1.8	2.38	1.3	1.39	1.2	.95
CH_2	2.1	2.7	1.6	2.63	1.2	.97	1.0	.91	1.0	.89	.91	.63
CH_3	2.4	2.7	1.7	2.5	1.4	1.0	1.0	.91	1.0	.79	.84	.44
$CH_3\underline{C}N$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$\underline{C}H_3CN$	5.2	4.49	3.0	3.08	2.8	2.06	2.3	2.11	2.2	1.81	2.0	1.4

* Instrument parameters differ; Lindmark data (+)

x B. Luke data

Mole ratios of gadolinium complex/ethylbenzene = 0.00358, 0.00716, and 0.01432 were prepared, but the sample would dissolve only upon addition of additional amounts of CH_3CN. Apparently, the complex without cyclohexane rings is more polar and thus less soluble in the less polar $CDCl_3$ solvent. These samples were, therefore, considered impractical from a solubility standpoint and no carbon-13 spectra were obtained.

An alternative experimental approach consisting of replacing the previous solvent system with DMSO-d_6 led to facile dissolution and the resulting ^{13}C data are presented in Table II.

Data in Table II indicate that quantitative integration differences due to changing the filter bandwidth from 4000 to 8000 Hz are substantial; 8000 Hz is the preferred bandwidth for quantitative results. In addition, by changing the solvent system to DMSO-d_6, the ring carbons and the ethyl carbons reach quantitative ratios at similar concentrations as opposed to the result in Table II. Good quantitative results (within 17-percent obtained at $4.26 \times 10^{-3} = (Gd^{3+})/(C_6H_5CH_2CH_3)$ (Bandwidth 8000 Hz, Table II). Above this ratio, the solubility limit of gadolinium is significant and severe broadening of the ^{13}C peaks is observed.

---

*While taking the earlier carbon ^{13}C spectra (Table III), it was discovered that quantitative results varied by changing the filter bandwidth. Filter bandwidth is an electronic filter which cuts down on noise in a spectrum, and can be represented as:

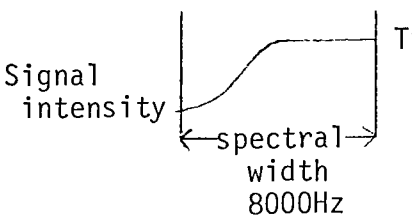


Fig. 10

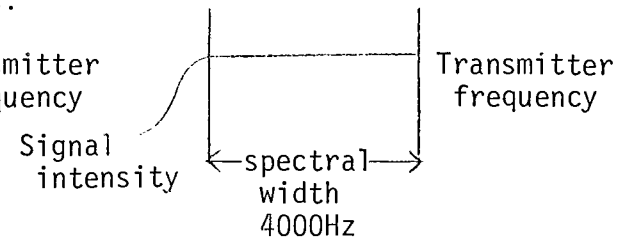
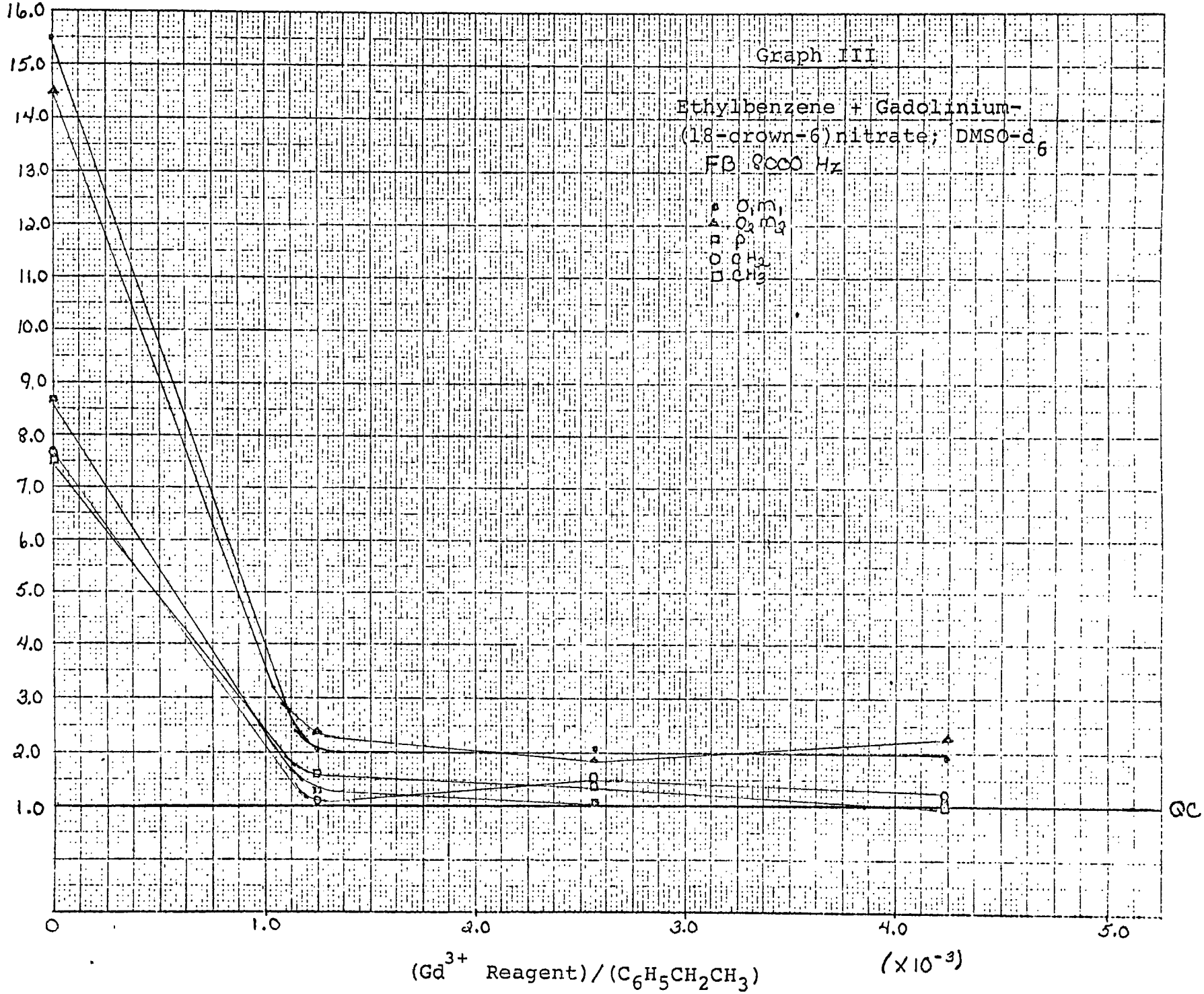


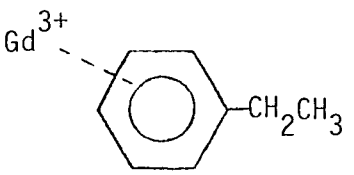
Fig. 11

This is of special interest in quantitative determinations since downfield peaks would be artificially attenuated by a 4000 Hz filter bandwidth. This problem isn't presented by an 8000 Hz filter bandwidth.


Graph III
Ethylbenzene + Gadolinium-(18-crown-6)nitrate; DMSO-d6
FB 8000 Hz
Intensity Observed/Intensity Expected for One Carbon
(Gd3+ Reagent)/(C6H5CH2CH3)
(X10-3)
QC
16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1.0
2.0
3.0
4.0
5.0

Since previous work with crown ethers complexed with lanthanide metals[31] clearly shows that the crown ether and lanthanide metal dissociate in solution, experimentation using gadolinium (III) without a crown ether was carried out. Gadolinium(III)nitrate pentahydrate, $Gd(NO_3)_3 \cdot 5H_2O$, was used as the relaxation reagent and ethylbenzene the substrate. Initially, we used the solvents used by Lindmark ($CH_3CH/CDCl_3$). The o_1m_1 and o_2m_2 integrations should approach two and the para, CH_2 and CH_3 carbons should approach one upon increasing additions of the relaxation reagent (Table IV).

The CH_3CN carbons are included and considered separately. These latter two signals do not undergo significant changes. It is interesting to note that quantitative results are only achieved at intermediate concentration ratio (7.4×10^{-4}) for the ethyl carbons, but quantitative results are not approached until the highest concentration ratio (59.3×10^{-4}) for the ring carbons. Obviously, a crown ether complexed to gadolinium is not needed for dissolution. The literature[16] suggests that the Gd^{3+} ion complexes less closely to the ethyl carbons than to the ring carbons. Our results may be consistent (Table IV) since the ethyl carbons do not achieve integration. This is consistent with the $1/r^6$ dependence of Gd^{3+} relaxation effects expected on T_1^e where r = the distance from the Gd^{3+} ion to the carbon in the complex:



A skewing of the Gd^{3+} ion away from the substituent side of the ring is consistent with steric skewing of the complex by the ethyl group.

TABLE II

Relative Integrations for Carbons of Ethylbenzene Treated with Gadolinium(18-crown-6)nitrate; DMSO-d_6

$\frac{(Gd^{3+})}{(C_6H_5CH_2CH_3)}$	0		1.23×10^{-3}		2.57×10^{-3}		4.26×10^{-3}	
Carbon								
QC	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
o_1m_1	8.57	15.5	2.09	2.05	1.96	2.08	2.16	1.91
o_2m_2	8.93	14.5	2.9	2.4	1.78	1.83	2.33	2.33
p	5.43	8.63	1.43	1.3	1.02	1.02	1.05	1.12
CH_2	3.21	7.63	1.54	1.15	1.43	1.52	1.40	1.26
CH_3	4.0	7.63	2.03	1.63	1.08	1.29	1.30	1.02
Filter Bandwidth (Hz)	4000	8000	4000	8000	4000	8000	4000	8000

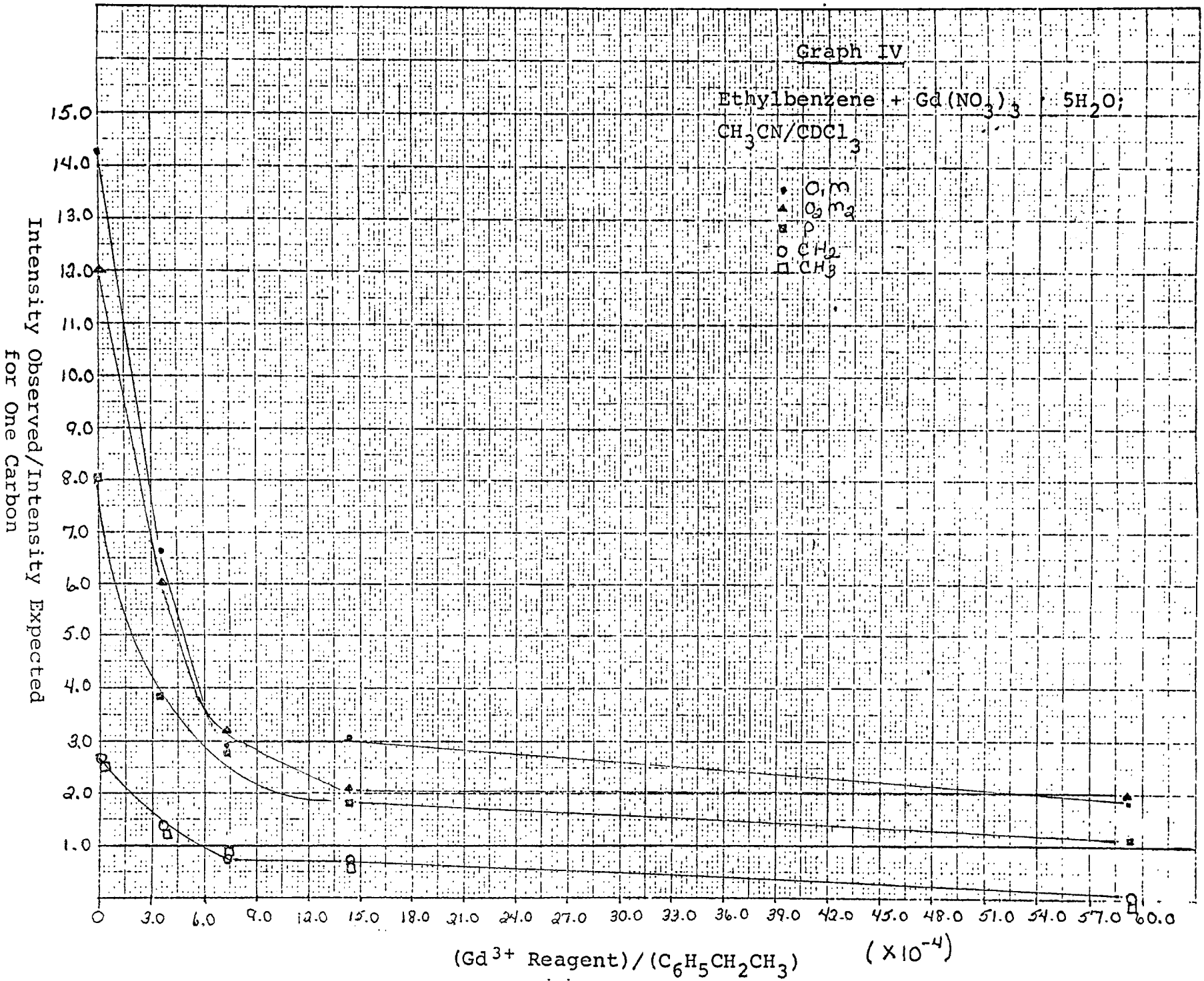
$(C_6H_5CH_2CH_3)$ = 0.0057M

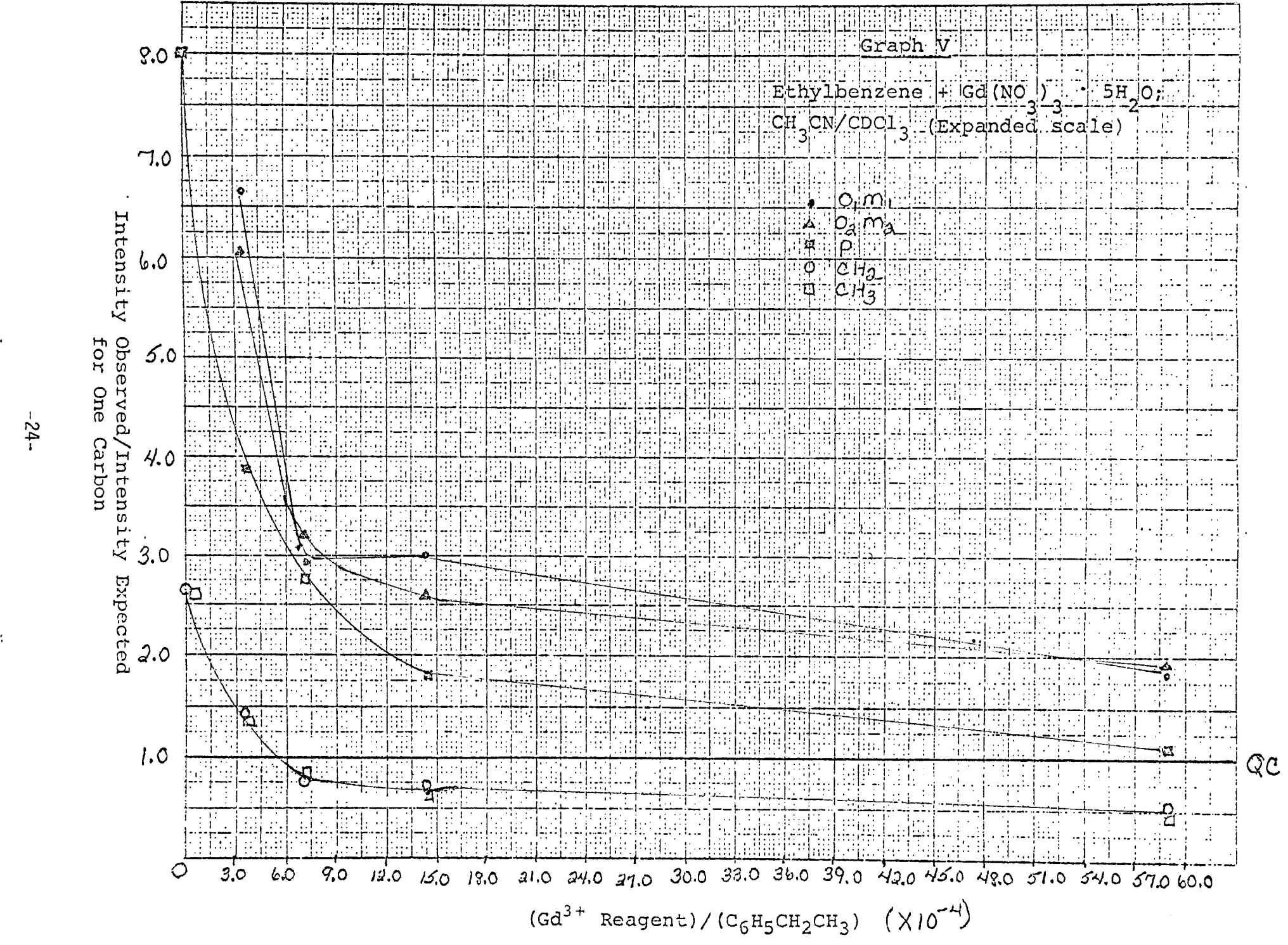
TABLE III

Relative Integrations for Carbons of CH_3CN and Ethylbenzene Treated with Gadolinium(III)nitrate pentahydrate; $CDCl_3/CH_3CN$; FB 8000 Hz

(Gd^{3+}) / $(C_6H_5CH_2CH_3)$	0	3.7×10^{-4}	7.4×10^{-4}	14.8×10^{-4}	59.3×10^{-4}
Carbon					
QC	1.0	1.0	1.0	1.0	1.0
o_1m_1	14.29	6.67	2.94	3.03	1.86
o_2m_2	12.0	6.07	3.23	2.58	1.96
p	8.0	3.87	2.74	1.79	1.16
CH_2	2.7	1.47	0.74	0.73	0.53
CH_3	2.7	1.47	0.81	0.67	0.37
$CH_3\underline{C}N$	1.0	1.0	1.0	1.0	1.0
$\underline{C}H_3CN$	4.49	1.45	1.12	1.51	1.64

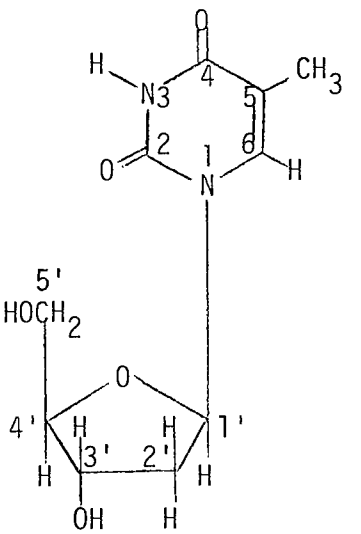
$(C_6H_5CH_2CH_3)$ = 0.0062M


Graph IV
Ethylbenzene + $Gd(NO_3)_3 \cdot 5H_2O$;
$CH_3CN/CDCl_3$
O_1
O_2
p
CH_2
CH_3
Intensity Observed/Intensity Expected for One Carbon
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
3.0
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
30.0
33.0
36.0
39.0
42.0
45.0
48.0
51.0
54.0
57.0
60.0
$(Gd^{3+}$ Reagent$)/(C_6H_5CH_2CH_3)$
$(\times 10^{-4})$


Graph V
Ethylbenzene + $Gd(NO_3)_3 \cdot 5H_2O$;
$CH_3CN/CDCl_3$ (Expanded scale)
$O_1 m_1$
$O_2 m_2$
p
CH_2
CH_3
Intensity Observed/Intensity Expected for One Carbon
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
3.0
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
30.0
33.0
36.0
39.0
42.0
45.0
48.0
51.0
54.0
57.0
60.0
$(Gd^{3+}$ Reagent$)/(C_6H_5CH_2CH_3)$ $(X10^{-4})$
QC

Since the quantitative results in previous work (Table II -- Filter Bandwidth 8000 Hz) with DMSO-d_6 were considered excellent, this solvent was used to study ethylbenzene substrate employing Gadolinium (III)nitrate pentahydrate as the relaxation reagent (Table V). These data clearly show that a crown ether complexed with Gadolinium is not needed to give quantitative results; the data at a 15.0 X 10^{-3} (Gd^{3+})/ ($C_6H_5CH_2CH_3$) ratio (highest ratio) show, within seven percent (7%), quantitative results.

After quantitative success with ethylbenzene using gadolinium(III) nitrate pentahydrate as the relaxation reagent and DMSO-d_6 as the solvent (the Gd^{3+} complex is greatly more soluble in DMSO-d_6 than $CDCl_3$/ CD_3CN), another substrate was studied: thymidine.



Thymidine

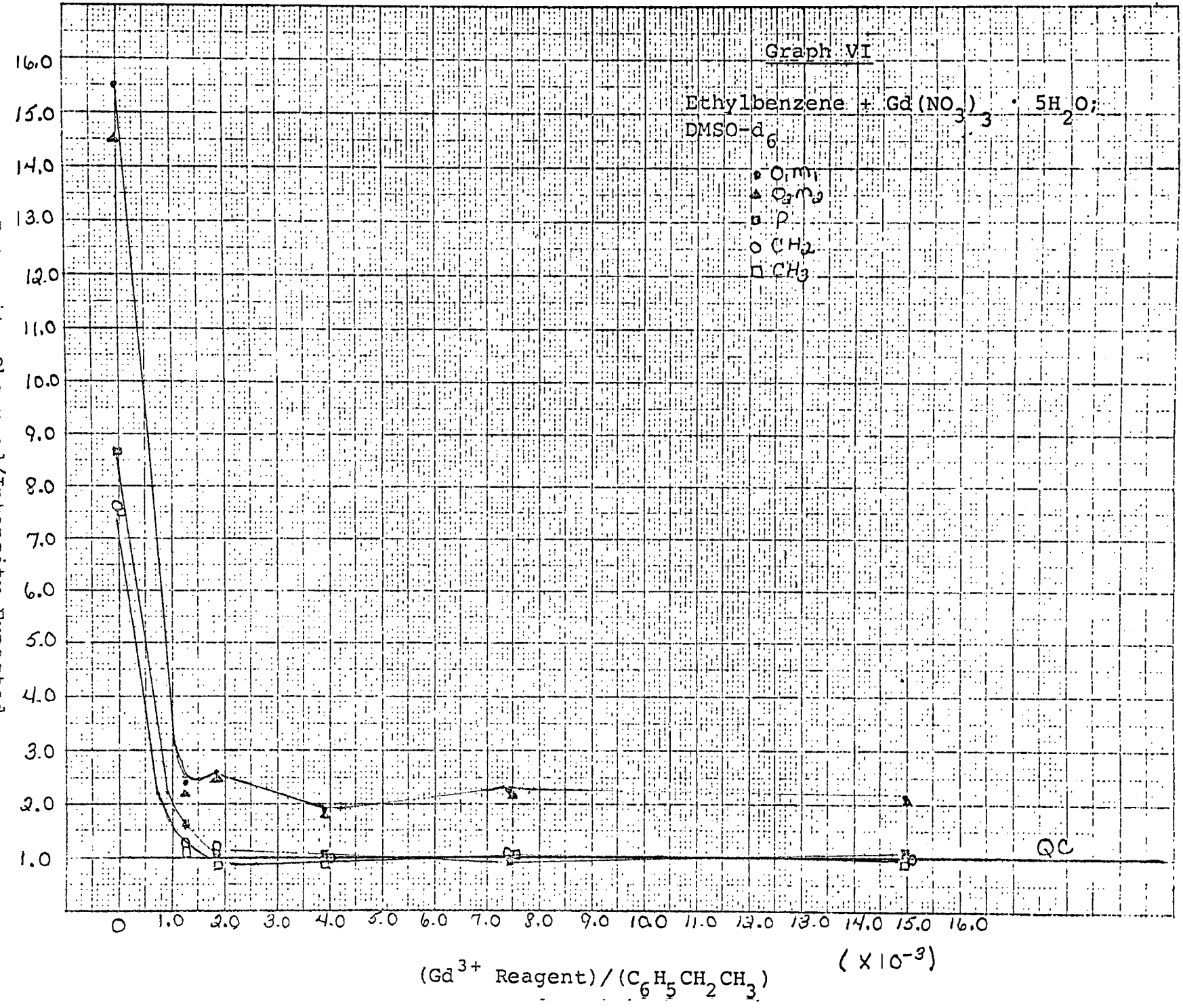
Fig. 12

TABLE IV

Relative Integrations for Carbons of Ethylbenzene Treated with Gadolinium(III)nitrate pentahydrate; DMSO-d_6; FB 8000 Hz

(Gd^{3+}) / $(C_6H_5CH_2CH_3)$	0	1.26×10^{-3}	1.85×10^{-3}	3.97×10^{-3}	7.49×10^{-3}	15.0×10^{-3}
Carbon						
QC	1.0	1.0	1.0	1.0	1.0	1.0
$o_1 m_1$	15.5	2.44	2.55	1.89	2.27	2.13
$o_2 m_2$	14.5	2.20	2.5	1.8	2.27	2.13
p	8.63	1.56	1.05	1.17	.98	1.04
CH_2	7.63	1.24	1.13	1.1	1.14	1.04
CH_3	7.63	1.1	.93	.96	1.0	.89

$(C_6H_5CH_2CH_3)$ = 0.006M


Graph VI
Ethylbenzene + Gd(NO3)3 · 5H2O;
DMSO-d6
Om1
Om2
p
CH2
CH3
QC
Intensity Observed/Intensity Expected for One Carbon
(Gd3+ Reagent)/(C6H5CH2CH3)
(X10-3)
16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1.0
2.0
3.0
4.0
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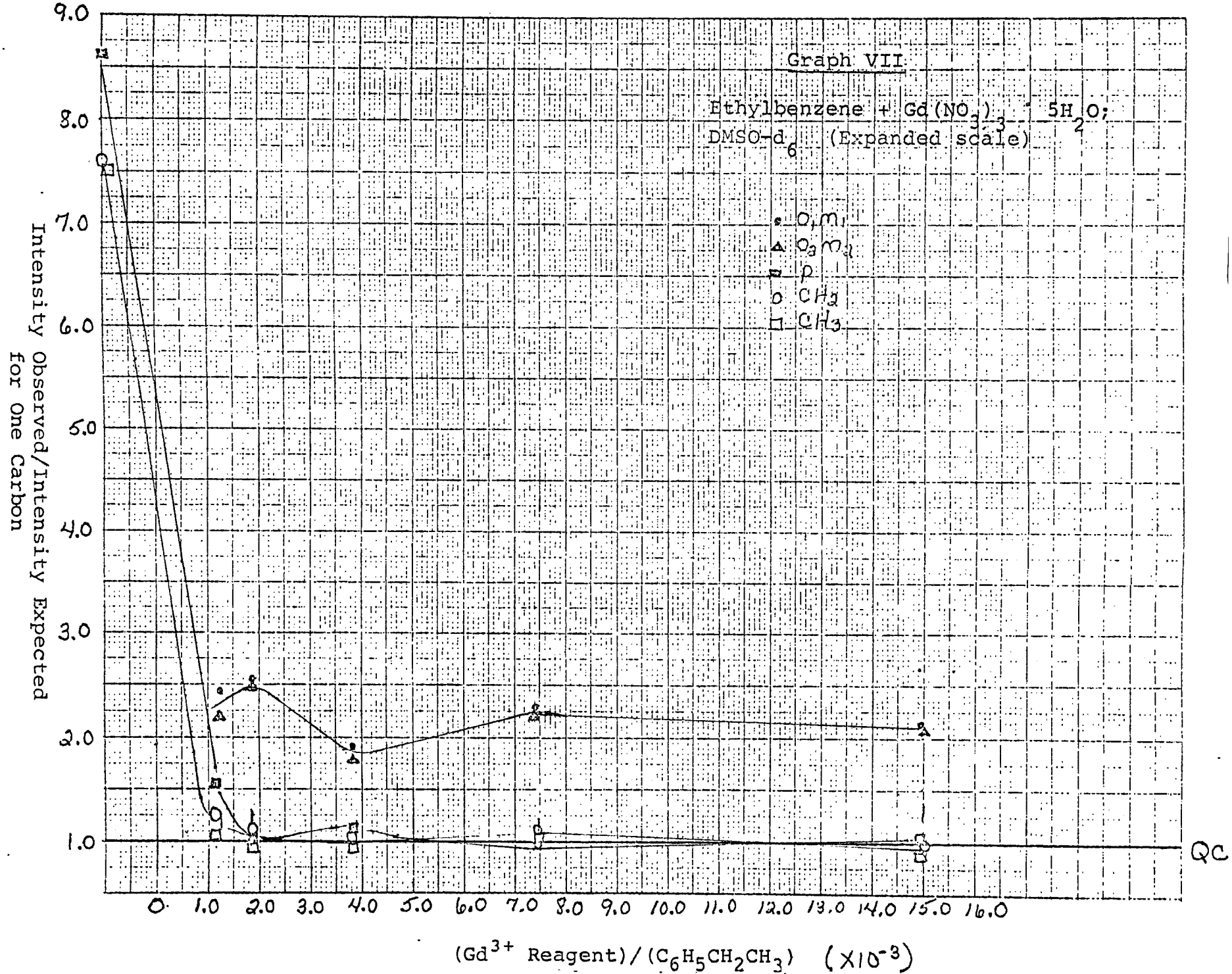
11.0
12.0
13.0
14.0
15.0
16.0


Graph VII
Ethylbenzene + Gd(NO3)3 · 5H2O;
DMSO-d6 (Expanded scale)
o, m1
o2 m2
p
CH2
CH3
Intensity Observed/Intensity Expected for One Carbon
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
(Gd3+ Reagent)/(C6H5CH2CH3) (X10-3)
QC

The reasons for studying this nitrogen heterocycle have been given in the historical section. Initial conditions involved using D_2O solvent and internal standard, DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate, $(CH_3)_3SiCH_2CH_2SO_3^-Na^+$) in D_2O solution with the thymidine. Results showed that upon continued addition of Gadolinium(III)nitrate pentahydrate, there was no significant change in intensities of the thymidine carbon signals. We do, however, obtain quantitative integrations for the carbon 13 signals of the internal standard, DSS (See Table X, Experimental Section). This implies we have competitive equilibria and the DSS competes for Gd^{3+} better than does thymidine ($K_1 >> K_2$):

$$(CH_3)_3SiCH_2CH_2CH_2SO_3^-Na^+ + Gd^{3+} \overset{K_1}{\rightleftharpoons} (CH_3)_3SiCH_2CH_2CH_2SO_3^-Gd^{3+} + Na^+$$

$$C_6H_5CH_2CH_3 + Gd^{3+} \overset{K_2}{\rightleftharpoons} C_6H_5CH_2CH_3 \cdot Gd^{3+}$$

Therefore, thymidine in D_2O was studied quantitatively without using DSS (Table V). Results indicate Gadolinium might be complexing preferentially with the sugar group, carbons 1' through 5'. This conclusion is drawn by observing that some of these carbons start to become quantitative (intensity approaches one), while the base carbons don't. But, in general, little change in relative intensity is observed on Table V, and thus, we have not connected the points on Graph VIII.

Since no integration change toward quantitative integrations of thymidine was observed using D_2O/DSS and since integrations in D_2O (no DSS) were not completely quantitative, the solvent system was changed to DMSO-d_6 containing no DSS (Table VI). These data also indicate that upon increasing additions of $Gd(NO_3)_3 \cdot 5H_2O$, the base carbons have made only modest changes toward quantitative integration, whereas the sugar carbons are virtually quantitative (within 20%) at 0.11245 molar ratio.

Graph VIII
Thymidine + $Gd(NO_3)_3 \cdot 5H_2O$;
DMSO-d_6
C-4
C-2
C-6
C-5
C-4'
C-1'
C-3'
C-5'
CH_3
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
.01
.02
.03
.04
.05
.06
.07
.08
.09
.10
.11
Intensity Observed/Intensity Expected for One Carbon
$(Gd^{3+}$ Reagent)/ (Thymidine)

TABLE V

Relative Integrations for Carbons of Thymidine Treated with Gadolinium(III)nitrate pentahydrate, D_2O; No DSS

(Gd^{3+}) / (Thymidine)	0	0.0280	0.0436	0.0736
Carbon				
C-4	0.256	0.125	0.0993	0.176
C-2	0.168	0.21	0.183	0.192
C-6	0.864	0.78	0.67	0.632
C-5	0.36	0.43	0.40	0.344
C-4'	1.0	1.0	0.88	0.792
C-1'	0.616	0.74	0.63	0.688
C-3'	0.944	0.96	0.84	0.8
C-5'	0.744	0.97	1.0	1.0
C-2'	0.808	0.96	0.92	0.976
CH_3	0.536	0.62	0.66	0.552

(Thymidine) = 0.0008M

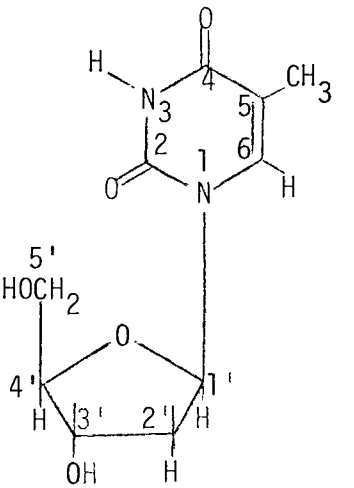


TABLE VI

Relative Integrations for Carbons of Thymidine Treated with Gadolinium(III)nitrate pentahydrate; DMSO-d_6

(Gd^{3+}) / (Thymidine)	0	0.0191	0.02103	0.0422	0.0515	0.06474	0.08042	0.11245*
Carbon								
C-4	0.33	0.28	0.14	0.43	0.40	0.46	0.67	0.67
C-2	0.51	0.28	0.36	0.43	0.47	0.62	0.60	0.53
C-6	0.54	0.56	0.71	0.64	0.67	0.62	0.80	0.80
C-5	0.70	0.44	0.43	0.64	0.60	0.69	0.80	0.73
C-4'	0.65	0.69	0.79	0.86	1.0	1.0	1.0	1.0
C-1'	0.74	0.69	0.64	0.64	0.73	0.69	0.73	1.0
C-3'	0.54	0.94	0.86	1.0	0.93	1.0	0.87	0.80
C-5'	0.44	1.0	1.0	0.86	1.0	1.0	0.87	0.87
CH_3	1.0	0.75	0.79	0.86	0.93	0.85	0.80	0.93

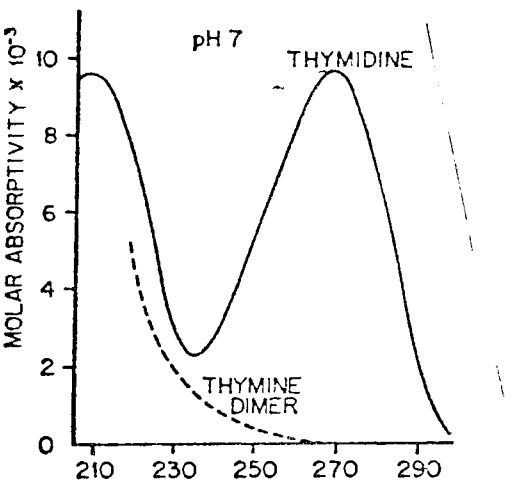
* increasing amounts of noise

(Thymidine) = 0.00082M

C-2' is masked by DMSO-d_6

One intended application of these Gd(III) additives is the quantitative determination of carbon signals of thymidine mixed with photochemically produced thymidine dimers. Before any photochemical irradiation was carried out, the wavelength of light needed for this photochemical experiment was determined by measuring the UV absorption spectrum of thymidine, λ_{max} = 258nm, experimental value; λ_{max} = 267nm, literature value.[30]

Fig. 13



The first approach to dimer formation was by internal irradiation, utilizing a Hanovia mercury arc lamp protected by a water cooled quartz immersion well. The solution to be irradiated surrounds the well and is exposed to the full output of the lamp. Solutions were prepared containing from 0.06M-0.008M thymidine with irradiation times up to sixteen hours. UV absorption measurements indicated approximately 15% dimer formation (refer to Historical). A similar procedure had been reported to show 14% dimer formation.[23] The remaining solution was lyophilized and a proton NMR spectrum indicated only the starting material thymidine.*

---

*A dimer ^1H NMR spectrum would show an approximate 0.4 ppm upfield shift of the methyl resonances compared with unreacted thymidine and an approximate 3.4 ppm upfield shift of the H_6 resonances of the dimer relative to the unreacted thymidine.[23]

Since there was such a small dimer conversion, a second approach to dimer conversion was considered. This consisted of using external irradiation by utilization of a Rayonet photoreactor.[29] The vessel to be irradiated is surrounded by lamps and is cooled by a fan. A concentrated solution of thymidine (0.19-0.25gm thymidine/3ml H_2O) (0.26M-0.34M) was prepared and placed in a quartz cuvette (1cm cell) for irradiation. Irradiation lasted approximately seven hours and samples were taken at one or two hour intervals. Maximum absorption changes were found to occur at times varying between three and six hours; after this time the dimer apparently began to reconvert to the monomer. Dimer conversion using this approach was apparently 32.85%-36.95%.

^{13}C spectral analysis detected no dimer formation. Probably not enough dimer was available to show up in the spectrum. Of the aqueous solution of thymidine, .26M, (0.19gm/3ml), approximately 37% was converted to (.07gm) dimers (0.19 X .37 = 0.07gm) or 0.048M. That is, the inconsistencies between UV absorption measurements and ^{13}C and ^{1}H spectra are a result of small absolute amounts of dimers (mixture of isomers) as the result of only modest amounts of conversion and the instrumental limitations of 0.05M (pure substance) for the CFT-20.* The signal/noise ratio is extremely low, but the carbon signals are visible for the monomer.

Since these dimer formation experiments were designed to test for mutagenic possibilities between or in DNA strands in living systems, and water has been reported to yield photo-dimers,[22,23] other solvents were not considered.

---

*The instrumental limitation was determined by making up solutions of decreasing concentrations of thymidine (for example, 2M, 1M, .05M, .025M) and accumulating pulses for a ^{13}C spectrum. When the signal/noise ratio at a certain concentration determined to be .05M, was so low that the thymidine peaks were no longer visible, this was considered the CFT-20 instrumental limitation.

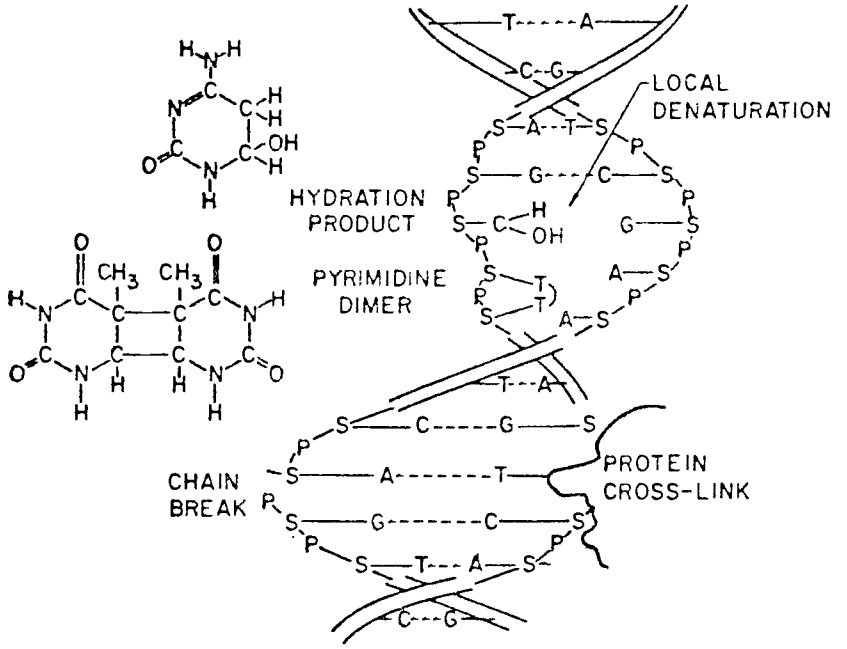


Figure 14. Schematic illustration of the various alterations found in DNA extracted from cells that have been irradiated with ultraviolet light. Some of these physical changes (e.g., denaturation) may be secondary effects resulting from photoproducts such as thymine dimers. The dimerized thymines are evidently unable to maintain hydrogen bonds with the adenine in the complementary strand. Other effects such as cross-linking to protein may involve new photoproducts yet to be characterized. Chemical structures for the cytosine hydrate and the thymine dimer are shown. (From "Ultraviolet Radiation and Nucleic Acid" By R. A. Deering. Copyright © 1962 by Scientific American, Inc. All rights reserved.)

Quantitative results of thymidine indicated that the sugar carbon integration approached quantitative integration (Table VI) while the nucleotide base carbons did not; therefore, we decided to thoroughly investigate the spin-lattice relaxation times and NOE's of the carbons in order to ascertain the differences in the carbons in the two portions of the thymidine system. These were of interest since the NOE factor should approach zero (0) with increasing additions of gadolinium(III) cation if the dipole-dipole

relaxation mechanism was completely eliminated. In addition, the spin-lattice relaxation times measured in the presence of Gd(III) should give an indication as to where the gadolinium was complexing in the molecule, as carbons close to gadolinium, in a Gd^{3+}-thymidine complex, would have spin-lattice relaxation times preferentially reduced. Gated decoupling was used to find the NOE values for thymidine. These data are given in Table VIII. As was expected,[14] the NOE is reduced for all carbons but not fully eliminated for every carbon. The NOE values for thymidine (Table VIII) are representative. Carbons with hydrogens attached, C-6, C-4', C-1', C-3', C-5', and CH_3, show more NOE than those carbons without hydrogens, as was expected. The more hydrogens attached to the carbons, the more NOE. In the presence of gadolinium, the NOE is shown to be sizeably reduced for every carbon with or without hydrogens attached.

TABLE VII

Nuclear Overhauser Enhancement Values (See Experimental Section for NOE Calculation) for Thymidine; DMSO-d_6

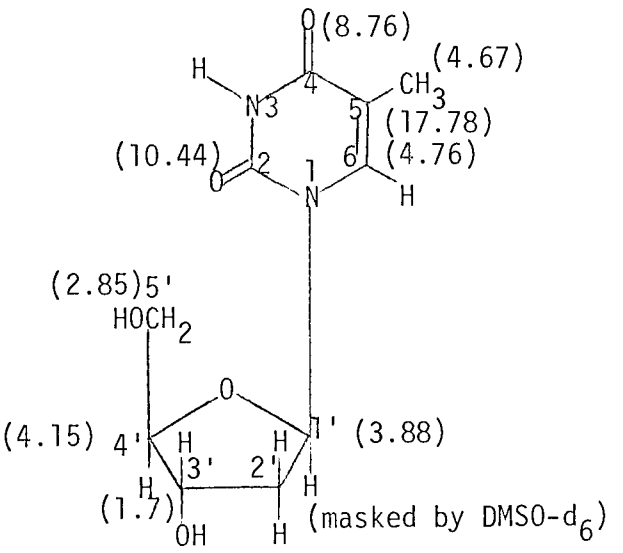
Carbons	Thymidine 0.004M	(Gd^{3+})/(Thymidine) = 0.065M	(Gd^{3+})/(Thymidine) = 0.053M
C-4	1.4	**	0.33
C-2	1.5	0	0.30
C-6	1.86	-0.125	0
C-5	1.2	0.56	0
C-4'	1.78	0.30	0.31
C-1'	1.86	0.29	0.42
C-3'	2.38	0.20	0.31
C-5'	2.33	0	0.21
CH_3	2.14	-0.57*	-0.53*

* Integration not reliable due to weak signal.
** Unable to be measured, due to noise.

An inversion-recovery (refer to Experimental Section) sequence was carried out to find the spin-lattice relaxation times of the thymidine carbons.[33] Time (t) values used were: 0.25, 0.5, 2.0, 5.0, 8.0, 15.0, 25.0, 35.0, and 50.0 seconds. The resulting spectra were integrated and the data developed by using $-\ln(M_0-M_t)$. The data was graphed, $-\ln(M_0-M_t)$ vs. t, and the slopes determined by a linear least squares method. The spin-lattice relaxation times (T_1) are the inverses of the slopes. The spin-lattice relaxation times (T_1) of thymidine (in seconds) are given below:

TABLE VIII

Spin-Lattice Relaxation Times (T_1) of Thymidine (Seconds) in DMSO-d_6

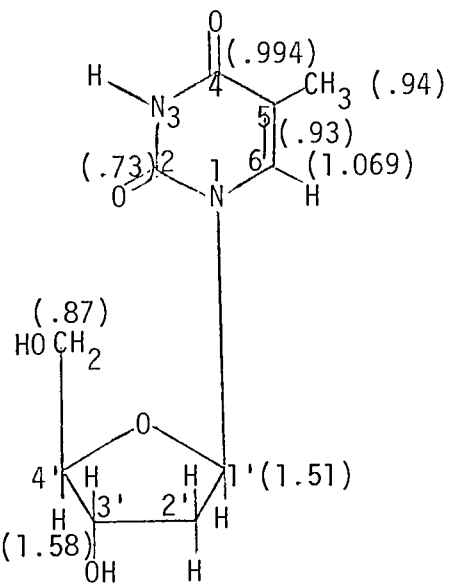


The spin-lattice relaxation times are needed for the pulse delay parameter in an NOE experiment. The longest T_1 in thymidine is 17.78 seconds, so 5 X 17.78 and adjusting for approximately 10% error, a suitable pulse delay is 97.8 seconds. Table IX gives the spin-lattice relaxation times (T_1) for thymidine in the presence of Gadolinium(III) nitrate pentahydrate, to indicate the lessening of the spin-lattice times and where gadolinium possibly complexes in a molecule. The position of

gadolinium complexing in thymidine seems to be deduceable from Table X. The greatest change in T_1's are C-5 (0.93 = T) and C-2 (0.73 = T_1), indicating gadolinium is acting most effectively here and therefore, complexing in this area, since nuclei close to the site of complexation have the shortest T_1 values.

TABLE IX

Spin-Lattice Relaxation Times (T_1) in Seconds
for (Gd^{3+})/(Thymidine) = 0.053M
in DMSO-d_6



It has been found recently[14] that an intensity ratio precisely corresponding to the number of adjacent carbon atoms can be reached only with infinitely high concentrations of a paramagnetic substance.

Therefore, by means of a linearized function,* it has been possible to give an extrapolation to the infinitely high concentration. In this way, quantitative ^{13}C NMR can be obtained routinely. The method does not require calibration and the standard deviation is $\pm$ 10% (relative).

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*The linearized function, after algebraic manipulation, becomes:

$$X = X_\infty - C \frac{X-X_0}{[E]} \quad \text{where:}$$

X = measured intensity ratio
X_∞ = value of "infinitely large concentration of $[E]$
C = empirical constant
$[E]$ = the paramagnetic substance concentration
X_0 = value of X in the paramagnetic substance free solution.

Therefore, when X vs. $\frac{X-X_0}{[E]}$ is plotted, the y-intercept is X_∞, the "infinitely" large concentration of the paramagnetic substance. Though we did not observe complete quantitative results in our studies, one could study these approaches to quantitative analysis[14] by various graphical plots and equations and obtain quantitative NMR results.

EXPERIMENTAL

Carbon magnetic resonance (^{13}C-NMR) spectra were obtained on a Varian CFT-20 (20 MHz) spectrometer and are proton spin-decoupled unless stated otherwise. Chemical shifts are expressed as parts per million (ppm) relative to the internal standard tetramethyl-silane (TMS) for the organic solvents (CH_3CN, $CDCl_3$ and DMSO-d_6) and to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for aqueous solvent (D_2O).

All solvents used (organic, aqueous) were deuterated, unless otherwise indicated.

Ultraviolet spectra were recorded on a Cary 219 spectrophotometer using a deuterium source lamp in the region 290-190nm in water.

DSS was omitted when using gadolinium(III)nitrate pentahydrate since quantitative experimentation with a solution of thymidine, D_2O, and DSS indicated the sulfonate salt carbons became quantitative, not the thymidine as was expected. (Refer to figure.) This inferred that the gadolinium was coordinating to the DSS. Literature[34] agrees, indicating that DSS coordinates to Ln^{3+} ions.

Data from spin-lattice relaxation studies were examined by use of a Linear Least-Squares-Fit program, written by Dr. William Hayles (Rochester Institute of Technology) for a Hewlett-Packard 9820 calculator.*

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*The Linear Least-Squares-Fit program is at the end of the experimental section.

Table X

DSS* + D_2O Quantitative Intensities Upon Addition of $Gd(NO_3)_3 \cdot 5H_2O$

$(\overset{a}{CH_3})_3Si\overset{b}{CH_2}\overset{c}{CH_2}\overset{d}{CH_2}SO_3^-Na^+$

Carbon	D_2O(3ml) + DSS Intensity Units	D_2O(3ml) + DSS Relative Intensity**	D_2O(3ml) + DSS + 0.1M $Gd(NO_3)_3 \cdot 5\ H_2O$ Intensity Units	D_2O(3ml) + DSS + 0.1M $Gd(NO_3)_3 \cdot 5\ H_2O$ Relative Intensity**
a	120	1.35	38	3.8
b	89	1.0	12	1.2
c	116	1.30	13	1.3
d	125	1.40	10	1.0

* Exact amounts were not recorded.

** Relative Intensity was calculated by dividing the intensity of each signal by the intensity of the weakest signal.

Ultraviolet irradiation of thymidine was carried out using a Hanovia high-pressure quartz mercury-vapor photochemical immersion lamp (Ace Glass, Inc.; Vineland, NJ; lamp catalog no. 6515-36; radiated energy: 202.7 watts) within a quartz immersion well apparatus (280ml) containing the solution to be irradiated or a Rayonet Photochemical Chamber Reactor Model RPR-100, containing RPR-2537 Å lamps with quartz cuvettes containing the solution to be irradiated.

The gadolinium(18-crown-6)nitrate and gadolinium(dicyclohexano-18-crown-6)nitrate were synthesized by David J. Long.[27] Gadolinium nitrate pentahydrate was purchased from the Alpha Division of Ventron. Thymidine and thymine were purchased from the Sigma Company. All other organic materials were commercial reagents used without any additional purification.

I. General Procedure for Quantitative ^{13}C Spectral Determination.

A known weight of substrate was placed in a ^{13}C-NMR tube (10mm O.D.). To this, a weighed amount of relaxation reagent and deuterated solvent (2.0-2.5ml.) DMSO-d_6, $CDCl_3$, or D_2O was added. The ^{13}C spectrum was obtained by pulsed FT analysis and the intensities of signals measured instrumentally.

This procedure (constant amount of substrate, and increasing amounts of relaxation reagent followed by obtaining the ^{13}C spectrum) was repeated until enough data, usually five points, was obtained to be plotted. A graph of the ratio of Intensity Observed to Intensity Expected (refer to Results and Discussion) for one carbon vs. (Gd reagent)/(substrate) was drawn and compared to the intensities of signals in a spectrum containing no relaxation reagent.

The CFT-20 parameter settings do not differ in comparison runs.

CFT-20

PARAMETER

SETTINGS

```
C13/??      0

FR      .5000
SW      4000
NT                  0
AT          0.819
PW           10
PD          0.000
DB        5552
CT                  0

TO           48
HF            1
RS            5
DM            1
DJ           50
NB    -    2000
AM            0
XY            0

SF    -       5.000
WP        4000
EP            0
WC        4000
EC            0
VS          100
RL          0.000
YY    -      40

PR            0
IN            0
AI            0
DC            0
TH           15
IS           50
SS            0
CA            1

TR            1
FM        5199
TT            0
LT          0.030
LI          0.000
NI            0
MT            0
DI            0

HS            0
NU         20.000
ST            0
AL          500
MA            0
DF          0.000
AP          0.137
DA          112
```

CFT-20 Parameter Settings include:

Bilter bandwidth: 4000 Hz or 8000 Hz*

Spectral width: 4000 Hz

Number of Transients: Varied with the experiment (Usually 250-8000 transients)

Acquisition Time: 0.819-1.023 seconds (the time that the free induction decay is sampled).

Pulse width: 10μs (this determines the flip angle. 20μs is the pulse width for a 90-degree flip angle.)

Transmitter Offset: Value varies with the solvent used.

Decoupler Offset: Value varies with the solvent used (determines the position of the decoupler in a proton spectrum at 80 MHz).

Pulse Delay: A delay in seconds before the rf pulse, and after the acquisition time. The magnitude depended upon the T_1 values of the substrate's carbons.

---

*Better quantitative results are obtained with the higher value. This is discussed further in Results and Discussion.

II. General Procedure for a Spin-Lattice Relaxation (T_1 Measurement) Experiment.

An inversion recovery[3] approach was used to determine spin-lattice relaxation (T_1) values. This consisted of a series of (Pulse delay - 180° inverting pulse - t - 90° observation pulse - acquisition time)n. (See Figure 8)

Where:

t = delay time between the 180° pulse and the 90° pulse

n = number of transients

The 180° pulse inverts the two ^{13}C energy level populations, producing a Boltzmann excess of nuclei in the higher energy level. Following this 180° pulse, the nuclei begin to relax to re-establish the normal Boltzmann distribution with excess nuclei in the lower energy state. The 90° pulse is applied after a delay time (t), which is varied in successive experiments. A free induction decay results from the 90° pulse.[3] In other words, the purpose of the 90° pulse is to obtain a record of the rate of decay of the 180° pulse.

In order for the inversion recovery pulse sequence to work efficiently, the pulse delay must be long enough for the spin to return to thermal equilibrium. After the signals are acquired for at least five different t-values (Figure 9) over a range that is expected to include the T_1, the spectra are recorded. The T_1 values for the individual carbon atoms are obtained by plotting $-\ln(M_0 - M_t)$ vs. t where:

M_0 = Integrated signal of the carbon from a normal spectrum of the substrate

M_t = Integrated signal of that carbon from a spectrum utilizing the (PD - 180° - t 90° - AT)n sequence.

The t-values are chosen over a range that's expected to include T_1. The slope of the line is $1/T_1$.

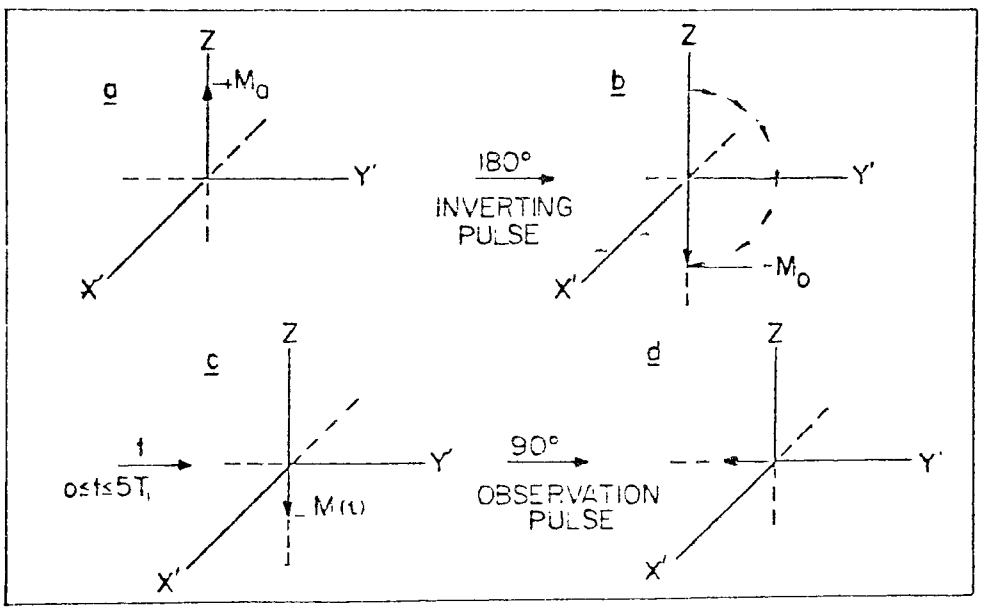

Fig.15: Inversion Recovery Pulse Sequence

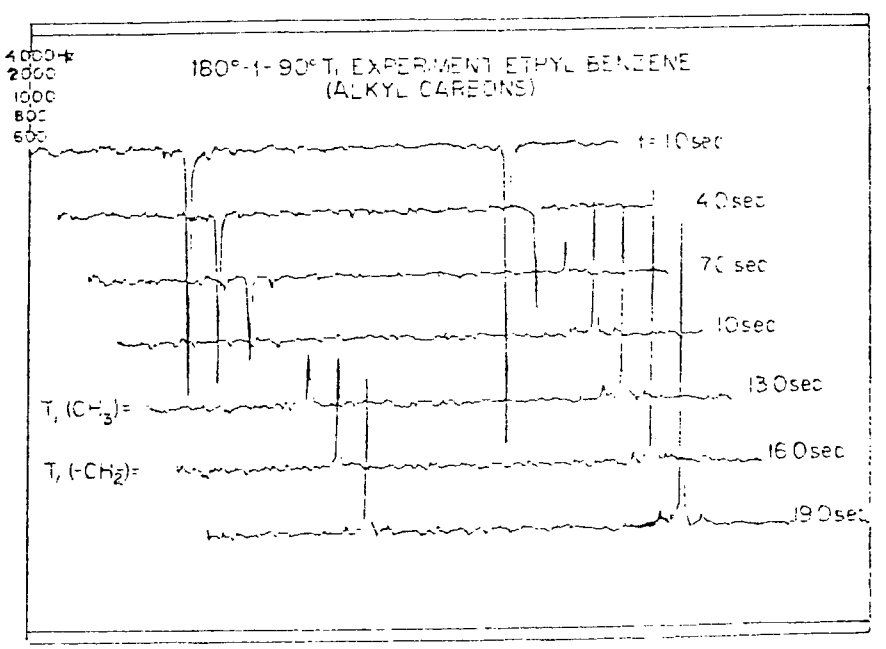

Fig. 16

Typical CFT-20 settings include:

Pulse Delay (seconds): A delay (5-300 seconds) before rf pulse and after the acquisition time.

Absolute Intensity (AI): AI = 0 means that the data plotted will be scaled to the tallest peak in the spectrum. Ex.: If the VS (Vertical Scale = 100, the largest peak will be plotted 100 chart units high. AI must equal one (1) for T_1 experiment as this allows the presentation of data relative to some absolute value from experiment to experiment.

180° Flip Angle: Inverting or the excitation pulse.

t: Time delay after the 180° flip angle.

III. General Procedure for Determining the Magnitude of the Nuclear Overhauser Effect.

NOE values can be determined using a NOE-suppression procedure.[28] Two pulse sequences are recorded, one with the decoupler on all the time (Decoupler Mode = 1) resulting in a completely decoupled spectrum with NOE; the other with the decoupler on only during the 90° pulse and acquisition time (Decoupler Mode = 3), resulting in a decoupled, but NOE-suppressed spectrum. The decoupler is off after the pulse. When the pulse is applied the spin states have their normal equilibrium populations. When the rf pulse is applied, the decoupler is turned on, (decoupling present during signal acquisition) and a decoupled spectrum with little or no NOE results. A pulse delay (5 X longest T_1) with the decoupler gated off must be inserted to ensure that the spin population have time to re-establish their equilibrium populations. The resulting spectra are transformed and integrated.

The NOE values are calculated by:

$$\text{NOE} = \left\langle \frac{\text{Integrated Value for a carbon of interest in the decoupled spectrum}}{\text{Integrated value for a carbon of interest in the NOE-suppressed spectrum}} \right\rangle - 1$$

IV. UV-Irradiation Experiment

A. Hanovia

The procedure for utilization of the Hanovia lamp and immersion well (apparatus described in general experimental section above) consisted of preparing a dilute aqueous solution of thymidine (0.06M - 0.008M) and placing this in the outer jacket of the Hanovia immersion well, with the mercury lamp in the inner jacket.

Irradiation time varied from 10-24 hours. Samples were taken after a given period of time to observe the dimerization progress.[29]

B. Rayonet

The procedure for utilization of the Rayonet apparatus (apparatus described in general experimental section above) consisted of placing a concentrated or dilute aqueous solution of thymidine (0.19 - 0.25gm thymidine/3ml H_2O) in a quartz cuvette and irradiating for up to eight hours. Samples were taken either every fifteen minutes or at hour intervals to monitor the dimerization process.

UV absorption measurements were made by combining 10µl of irradiated solution with 5ml of 50mM Na_2HOP_4 buffer solution and 5ml of 50mM KH_2PO_4 buffer solution to keep the pH at approximately seven. UV scans were made from 290 to 190nm. Unreacted thymidine absorbs at λ_{max} = 267nm (ε = 9.3 X 10^3, pH 7, water).[30]

LINEAR LEAST-SQUARES-FIT
PROGRAM PRINT-OUT

```
 Ø:  1→R8; PRT "CONSTANTS?"; SPC 3; STP; TRC
 1:  NOR; SPC 6; TBL 6
 2:  PRT "OUTPUT FORM"; SPC; PRT "Z=Ø FIXED PT", "Z=1 EXPONENTIAL"; SPC
 3:  ENT Z; IF FLG 13; CFG 13; JMP Ø
 4:  FXD Ø; PRT Z; SPC 3
 5:  IF Z=Ø; 0→R9; GTO +2
 6:  1→R9
 7:  PRT "DECIMINALS IN", "OUTPUT"; SPC
 8:  ENT "DECIMALS", Z; IF FLG 13; CFG 13; JMP Ø
 9:  PRT Z; SPC 3; Z→R1Ø
1Ø:  Ø→Z; PRT "DATA LIST; X,Y"
11:  Ø→RZ; JMP (1+Z→Z) = 6
12:  FXD R1Ø; CFG 13
13:  IF R9 = 1; FLT R1Ø
14:  IF FLG 3 = Ø; ENT X; IF FLG 13; GTO "BRCH"
15:  IF FLG 3; ENT Y; IF FLG 13; GTO "BRCH"
16:  IF FLG 3; PRT Y, (Y - R7)/R6; SPC; GTO -1
17:  PRT X
18:  IF FLG 2; PRT XR6+R7; SPC; GTO -4
19:  ENT Y; IF FLG 13; CFG 13; JMP Ø
2Ø:  PRT Y
21:  IF FLG 1; GTO "DEV"
22:  SPC
23:  RØ + R8→RØ
24:  R1 + XR8→R1
25:  R2 + YR8→R2
26:  R3 + XYR8→R3
27:  R4 + XXR8→R4
28:  R5 + YYR8→R5
29:  GTO 14
30:  "DEV"
31:  Y - XR6 - R7→Y; PRT Y; SPC
```

LINEAR LEAST-SQUARES-FIT
PROGRAM PRINT-OUT
(Continued)

```
32:  GTO 14
33:  "BRCH"
34:  IF FLG 13; SPC 4
35:  Ø→Z; TBL 6
36:  ENT "EXIT?", Z; IF Z; GTO "END"
37:  ENT "NO. POINTS?", Z; IF Z; Ø→Z; PRT "N=", RØ
38:  ENT "ADD DATA?", Z; IF Z; PRT "ADDED", 1⇒R8; GTO 12
39:  ENT "DELETE DATA?", Z; IF Z; PRT "DELETED";  1⇒R8; GTO 12
40:  R3 - R1R2/RØ→R11
41:  R4 - R1R1/RØ→R12
42:  R11/R12→R6
43:  (R2 - R1R6)/RØ→R7
44:  ENT "LEAST SQRS LINE?", Z; IF Z; GTO +5
45:  ENT "X IN: LSQ Y OUT", Z; IF Z; PRT "X, CALCD Y"; SFG 2; GTO 12
46:  ENT "Y IN: LSQ X OUT", Z; IF Z; PRT "Y, CALCD X"; SFG 3; GTO 12
47:  ENT "X,Y IN:Y DEV OUT", Z; IF Z; SFG 1; PRT "X,Y, Y DEVIATION";
     GTO 12
48:  CFG 13; GTO "BRCH"
49:  FLT 3
5Ø:  IF R9=1; IF R1Ø> 3; FLT R1Ø
51:  R5 - R2R2/RØ→R13
52:  (R13 - R11↑2/R12)/(RØ - 2)→R14
53:  R11/√(r12R13)→R15
54:  R14/√R12→ R16
55:  R14√(1/RØ + (R1/RØ)↑ 2/R12)→ R17
56:  PRT "SLOPE =", R6
57:  PRT "INTERCEPT =", R7
58:  PRT "CORRELATION COEF", R15
59:  PRT "SIGMA (SLOPE) =",√ R16
6Ø:  PRT "SIGMA (YCEPT) =",√R17
61:  GTO "BRCH"
62:  "END"; PRT "END"; SPC 8; END
```

R257

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